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Probiotic potential of *Lactobacillus fermentum* G-4 originating from the meconium of newborns

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Abstract: The present study was dedicated to determining probiotic potential of a human isolate G-4, originated from meconium. The isolate was identified using morphological, physiological and biochemical assays and molecular method based on *16S rRNA* gene sequencing. In order to evaluate its probiotic properties *in vitro* tests were performed: the survival in simulated gastrointestinal conditions, adhesion to hexadecane, and antimicrobial activity. Safety aspects of the isolate were examined by testing toxicity, gastrointestinal tolerance and bacterial translocation *in vivo*, as well as hemolytic activity *in vitro*. The isolate G-4, identified as *Lactobacillus fermentum*, showed viability in artificial gastric and intestinal juice (low degree of cell viability reduction for 0.69 and 1.30 logCFU mL^{-1} units, respectively), moderate adhesion to hexadecane (39±2.1 %), and antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serotype Abony and *Clostridium sporogenes*, due to production of lactic acid (9.80 g L^{-1}). No signs of toxicity, bacterial translocation, hemolytic activity, were observed.

Keywords: *Lactobacillus fermentum*; meconium; probiotic; safety.

INTRODUCTION

Probiotics are live microorganisms that, when administered in an adequate amount, confer health benefits to the host.[1] The guidelines for the evaluation of probiotics recommends that every potential probiotic strain be correctly identified using phenotypic and genotypic methods, followed by various tests to investigate its survival ability and functional properties.[1] Probiotic strains, such as

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Lactobacillus, *Bifidobacterium* and *Streptococcus*, have long histories of safe use and are considered as generally recognized as safe (GRAS) microbes.[1] According to the FAO/WHO guidelines (FAO/WHO, 2002), the main criterion for probiotic selection and application is the survival of the bacterial cells during their passage through the gastrointestinal tract (GIT) of the host. Acidity and the presence of bile salts and pancreatic enzymes are some of the major stressors that an orally taken probiotic encounters in the GIT.[2] In order to realize its beneficial effect, a probiotic strain, apart from being able to survive, has to be able to adhere to human intestinal epithelial cells and, at least temporarily, colonize the different part of the GIT.[3] Important properties of a potential probiotic also involve auto-aggregation, cell surface hydrophobicity, absence of toxicity and translocation.[4,5] Additionally, antimicrobial activity against different pathogens is another important aspect of the probiotic *Lactobacillus* spp., which is associated with alteration of the intestinal microbiota and has been used to suppress pathogen growth.[6] This is accomplished by the production of antimicrobial compounds (organic acids, hydrogen peroxide, diacetyl and bacteriocins) by the probiotic and also by its competition for nutrients and their interference with human intestinal pathogen invasion.[6,7] Recent studies were directed at further dissemination of the applications of strain-specific probiotic effects in medicine (neurology, immunology, endocrinology, *etc.*) due to their production of bioactive metabolites.[8] Probiotic strains that are commercially applied most commonly belong to different *Lactobacillus* spp. isolated from oral, vaginal mucosa and intestines (especially from rectal mucosa of healthy people), breast milk and cheeses.[9] A small number of studies related to the evaluation of the safety of probiotic *Lactobacillus* species originating from human milk and baby meconium have been reported.[10,11] Particularly, an infant formula supplemented with *L. fermentum* CECT5716 was examined to evaluate safety and tolerance and investigate the effects of a follow-on formula containing the same strain on the incidence of infections in infants between 6 and 12, and 0 to 6 months of age.[10,11]

The aim of the present study was to identify and evaluate the probiotic potential of a new intestinal isolate originating from newborn meconium, in order to assess it as a probiotic candidate.

EXPERIMENTAL

Indicator strains, media, and growth conditions

Staphylococcus aureus ATCC 6538P, *Escherichia coli* ATCC 8739, *Salmonella enterica* subsp. *enterica* serotype Abony NCTC 6017, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Clostridium sporogenes* ATCC 19404, *Candida albicans* ATCC 10231, were grown on the following selected media: Baird Parker agar, MacConkey agar, deoxycholate lactose agar, cetrimide agar, tryptone soya agar, B. cereus selective agar, sulfite agar and Sabouraud dextrose agar, respectively. *C. albicans* was incubated at 25 °C for 72 h, while bacterial cultures were incubated at 37 °C for 48 h. The media were manufactured by Torlak, Serbia.

Isolation of bacteria from the collected meconium samples

Samples of fresh meconium from 10 donors (infants born in the University Hospital in Belgrade, Serbia) were collected in sterile dry plastic containers and stored at 4 °C, until processing. Isolation of lactic acid bacteria (LAB) was performed according to Al Atya *et al.*[12] About 1 g of meconium was resuspended in 10 mL of sterile 0.05 M potassium phosphate buffer with cysteine (0.05 %) and homogenized for 2 min. A set of tenfold dilution from 10^{-1} to 10^{-6} was made in the phosphate buffer. Subsequently, 100 µL of each dilution was smeared on the surface of MRS (de Man–Rogosa–Sharpe) agar medium (Biokar, France) and gently poured. The inoculated plates were cultivated anaerobically, at 37 °C for 72 h. The suspected individual colonies were further characterized. These bacterial isolates were repeatedly sub-cultured to obtain pure cultures. The isolated pure cultures were further maintained at 4 °C on MRS agar.

Preliminary testing – morphological, cultural and physiological characteristics

The individual colonies obtained from the preliminary cultivation were characterized using standard protocols for identification of LAB: Gram-staining, cell morphology, catalase reaction (6 % hydrogen peroxide solution), growth in MRS broth at different temperatures (15, 30, 37, 45 and 50 °C) for 5 days, growth in MRS broth of different salt concentrations (2, 4 and 6.5 % NaCl) for 5 days at 37 °C, CO_2 production from glucose in a tube containing inverted Durham's tubes, L-arginine hydrolysis, liquefaction of gelatin and growth in 10 % skimmed milk medium.[13] All tests were performed in triplicate.

Analytical profile index (API) identification

One isolate was chosen from the 20 preliminary selected isolates and designated as G-4. This isolate was primarily identified at the species level using the API 50 CH System and 50 CHL medium (BioMérieux, France), according to the manufacturer's instructions. The results were recorded after 2 days of incubation at 37 °C and evaluated with identification software Vitek (BioMérieux, France).

Hemolytic activity

The hemolytic test was preformed according to Lombardi *et al.*[14] by streaking selected lactobacilli on Columbia Blood agar supplemented with 5 vol. % of human or sheep blood. A positive result is indicated by a clear zone around the colonies (as *β*-hemolysis positive) and no zone indicates a negative result. Greenish zones around the colonies were interpreted as *α*-hemolysis and taken as negative for the assessment of hemolytic activity. The test was performed in triplicate.

Molecular identification

Lactobacilli genomic DNA was isolated using the QIA DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). PCR amplicons were generated using Taq polymerase (Pharmacia, Vienna, Austria), according to the supplier's instructions. PCR products were analyzed on 1 % agarose gel and purified using the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). Species determination was realized by PCR, using primers complementary to 16S rDNA: UNI16SF (5'-GAG AGT TTG ATC CTG GC-3) and UNI16SR (5'-AGG AGG TGA TCC AGC CG-3'). PCR amplifications were performed using the Gene Amp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) and Taq polymerase (Pharmacia, Vienna, Austria). The resulting PCR amplicons were purified with the QIAGEN PCR purification kit (QIAGEN GmbH Hilden, Germany), following the manufacturer's instructions. Sequencing was performed in the Central Service of Macrogen (Macrogen, Seoul, South Korea) using the dideoxynucleotide DNA chain termination method. The BLAST algorithm

(http://www.ncbi.nlm.nih.gov/BLAST; RID: 1138633900-27581-131272740575.BLASTQ4) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Screening of probiotic potential

Resistance to artificial gastric and intestinal fluids. To screen the potential probiotic properties of G-4, its survival in artificial gastric juice (AGJ) and bile salt solution (BSS) were examined. In the first step, the test of bacterial survival in AGJ was performed *in vitro* according the literature.[3] Thus, 10 mL of cell suspension (1×10^8 CFU mL^{-1}) was added to 90 mL of AGJ (0.03 M NaCl, 0.3 % pepsin at pH 2.0 adjusted with 10 M HCl (Sigma–Aldrich)), and incubated with gentle agitation (58 rpm) in a shaker (Adolf Kühner, Switzerland) to simulate peristalsis. The cell suspension was obtained by washing and suspending the cells harvested from 10 mL total culture by centrifugation in sterile PBS. Aliquots of cells in AGJ were taken and their viability was determined by the enumeration of viable cells after 0, 60 and 120 min. The bacterial survival is expressed with reference to the initial bacteria count. In the second step, the effect of BSS on bacterial survival was studied by suspending the harvested cells in PBS buffer (0.01 M K_2HPO_4, 0.01 M KH_2PO_4 and 0.15 M NaCl) containing 0.5 % bovine bile salts and adjusting the pH to 8.0 with 1 M NaOH. The suspensions were incubated at 37 °C for up to 2 h with gentle agitation (58 rpm) in order to ensure homogenization. The sampling was performed at 0, 30, 60, 90 and 120 min, and the cell viability determined. Bacterial survival is expressed as log (CFU mL^{-1}) difference between the initial cell number and cell number after the treatment.

Microbial adhesion to hexadecane (MATH) in vitro *test.* A culture was grown on a stationary phase and then centrifuged (5000*g*, 10 min) in order to separate the fermentation liquid and the biomass. The biomass was washed twice in 0.1 M KNO_3 (pH 6.2) and resuspended, so that the optical density (*OD*) at 600 nm was 0.4 (value defined as A_0). To the cell suspension (1.2 mL) was added 0.2 mL of hexadecane as solvent. After 10 min of incubation at 25 °C, the biphasic system was strongly mixed for 2 min and then incubated at 25 °C for 15 min, after which the aqueous phase was separated and the *OD* of the treated cells was measured at 600 nm (value defined as A_1). The percentage of cells bound to solvent, θ, was calculated according to the formula: $100(1 - A_1/A_0)$. Values of θ of 0–35 indicate low, 36–70 medium and 71–100 high hydrophobicity.[15] The standard control was strain *L. rhamnosus* ATCC 7469.

Detection of antimicrobial activity

For the evaluation of antimicrobial activity of isolate G-4 agar-well diffusion assay (AWD) was used.[16] To detect the antimicrobial activity, the following indicator strains were used: *S. aureus* ATCC 6538P, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. enterica* subsp. *enterica* serotype Abony NTCC 6017, *B. subtilis* ATCC 6633, *B. cereus* ATCC 11778, *C. sporogenes* ATCC 19404, and *C. albicans* ATCC 10231. The overnight culture of the indicator strains were mixed at 1 vol. % with melted nutrient agar (corresponds to 1×10^6 CFU mL^{-1}), poured into sterile Petri dishes and after solidification, a well of 6 mm diameter was made in the center. Separation of cells from the supernatant of an overnight G-4 culture (18 h) was performed by centrifugation for 20 min at 4000 rpm (Universal 320/320 R, Hettich, Germany), after which the supernatant was filtered through sterile 0.45 μm Millipore filter. Cell-free aliquots of the filtrate (100 μL) were added into the wells. The plates were first stored at 4 °C for 2 h in order to allow the test material to diffuse through the agar and then incubated for 18 h at 37 °C for bacterial strains and at 25 °C for *C. albicans*. After incubation,

the clear zone of inhibition around the wells was measured. Additionally, in order to determine the nature of antagonistic activity of G-4, supernatant neutralized with 1 M NaOH was further tested by the same method against the same test strains. All tests were performed in triplicate.

Qualitative and quantitative analysis of organic acids

Organic acids were determined by qualitative and quantitative HPLC analysis (HP1100, Hewlett Packard, Palo Alto, CA, USA) with an ion exchange column (Supelco gel C-610H, Supelco, USA) using 0.1 % H_3PO_4 as the mobile phase. The flow rate of the mobile phase was 0.5 mL min^{-1} and the absorbance at 210 nm was measured by a diode array detector (DAD 1100, Hewlett Packard). Verification of the metabolite was determined by HLPC (LC-6A, Shimadzu, Kyoto, Japan), with the same column, mobile phase and flow rate, and a refractive index detector (RID, 9100 Varian Inc, Palo Alto, CA, USA). The reproducibility was checked by determination in triplicate. The system suitability and linearity for concentration of lactic acid was checked by standard organic acid kit (cat. No. 47264, Supelco Inc, Bellefonte, PA, USA).[17]

In vivo *safety study*

Preparation of culture for the in vivo *study.* For the long term preservation, *L. fermentum* G-4 was lyophilized and stored at –20 °C. After recovery at 36 °C for 30 min, the culture was inoculated into MRS liquid medium and incubated for 18 h at 36 °C in an anaerobic workstation. The biomass was harvested by centrifugation at 5000 rpm for 20 minutes at 4 °C, washed, and finally resuspended in sterile physiological solution in order to obtain 1×10^9 CFU mL^{-1}.

Experimental animals and housing conditions. Young adult NMRI Han mice (5–6 weeks old), both masculine and feminine, the weight of which was between 18 and 22 g (obtained from Galenika a.d. R&D Institute, Center for Biomedical Research) were placed in makrolon cages. Eight cages, each accommodating five mice of the same sex per cage, were placed in chambers with a regulated temperature of 23±2 °C, a relative humidity of 60±5 % and provided 12 h light/dark cycles. Before the start of the dosing, all mice were quarantined for a period of 7 days during which mass gain and any gross signs of illness or injury were analyzed. The mice were fed with commercial pellet food for small rodents (the Veterinary Institute, Subotica, Serbia) and drinking water *ad libitum*. The cages were maintained clean, and sterile bedding was changed weekly. This study was conducted in accordance with the regulations and standards: The Law on Experimental Animal Treatment – Official Gazette of RS No 41/2009 and European Directive 2010/63/EU, European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.[18,19] The experiment was approved by the Animal Ethics Committee - Galenika a.d. No. 05/11.

Abnormal toxicity test. Abnormal toxicity test was implemented in accordance with general requirements Ph. Eur 6.0.[20] The test is considered successful if none of the mice dies within 72 h after appliance. The dose was determined in accordance to human body mass of 70 kg and should correlate with the daily probiotic dose of 1×10^{11} CFU kg^{-1}. Daily, the experimental animals received lactobacilli resuspended in 0.5 mL of saline. Behavioral pattern, changes in the skin and fur, eyes and mucous membranes and excreta, as well as feed and water consumption were observed and examined.

Gastrointestinal subacute local tolerance. Ten NMRI Han mice (six weeks old, weighing 18–22 g) were dispensed by gastric intubation 1.4×10^7 CFU mL^{-1} of G-4 every day during the experimental period of 7 days. The control group consisting of 5 mice was given 1 mL of

saline by gastric intubation. Both groups had the same housing conditions during this period and free access to food and water. During the experimental period, all mice were surveyed every day and analyzed for general signs of toxicity: changes in the skin and fur, eyes and mucous membranes, excreta, behavioral pattern, and food and water consumption. After finishing the treatment, animals were sacrificed by cervical dislocation after ether anesthesia, and then a macroscopic examination of inner organs was conducted.

Bacterial translocation. Translocation of bacteria to blood and tissues was assessed according to Zhou *et al.*[21], in the same animals. Briefly, one drop of blood (10 μL) was placed onto the surface of MRS agar plates and incubated at 37 °C anaerobically to detect bacteremia. The excised spleen, liver and kidney were homogenized in 5 mL sterile saline solution, and 100 μL of tissue suspensions plated on MRS agar plates. The plates were then incubated anaerobically, at 37 °C for 48 h.

RESULTS AND DISCUSSION

Meconium (10 different samples) from healthy two-day old newborns was used for the isolation of newly isolated lactobacilli strains. Two from ten samples were sterile – no colonies had grown even after 72 h of incubation, while from other eight, 31 colonies were preliminary isolated (based on colony morphology) and selected for further testing in order to isolate LAB.

Isolation and preliminary characterization of LAB isolate

Lactobacilli are generally isolated on rich media, such as MRS that is routinely used for the isolation and counting of lactobacilli from most (fermented) food products. The addition of a reducing agent, such as cysteine, 0.05 %, to MRS improves the specificity of this medium for *Lactobacillus* spp. isolation.[21] Preliminary selection of LAB using standard protocols gave one isolate, designated as G-4 (Supplementary material to this paper, Table S-I). The production of CO_2 by isolate G-4 indicated that it belongs to the group of heterofermentative lactobacilli. Based on preliminary microbial tests, biochemical identification was performed by API 50 CHL kit (BioMerieux, France). According to the profile of fermentation of carbohydrates, the isolate showed greatest similarity to bacteria belonging to *L. fermentum* (Table S-II of the Supplementary material to this paper).

Hemolytic activity

In the test, no zones of hemolysis (clear or greenish) were observed, which indicates that the isolate exhibits no hemolytic activity. The obtained results are in agreement with characteristics of bacteria belonging to *Lactobacillus* genus.[14]

Molecular identification

Based on phenotypic and molecular characterization (Supplementary material, Table S-III), the bacterial isolate denoted as G-4 was identified as *L. fermentum* and marked among the laboratory isolates as *L. fermentum* G-4. The strain is deposited in bacterial collection of Galenika a.d.

Screening of L. fermentum *G-4 probiotic potential*

In vitro and *in vivo* tests were performed in order to determine potential probiotic properties of the isolate. Probiotics acting in the GIT must be resistant to gastric acid digestion and to bile salts to reach the intestinal intact, and they should be nonpathogenic.[22]

Resistance to artificial gastric and intestinal fluids

Preservation of the microbiological balance in the human gastrointestinal tract is necessary characteristic of a probiotic culture, together with GRAS profile, tolerance to the GIT conditions, ability to adhere to mucus and/or epithelial cells, antimicrobial activity against pathogens and susceptibility to antibiotics.[7] These are of great importance since disturbed equilibrium, especially between *Lactobacillus*, as one of the most important Gram-positive, and other, mainly Gram-negative bacteria, might result in the occurrence of various diseases. *L. fermentum* G-4 showed a high degree of survival in AGJ and in the solution containing 0.5 % BSS. After 120-min exposure to AGJ, the number of viable cells had decreased by 0.69 log(CFU mL^{-1}). The cells that survived AGJ conditions were then exposed to a bovine bile salts solution and after 120-min exposure, the number of viable cells had decreased by 1.43 log(CFU mL^{-1}) (Table I). The survival of *L. fermentum* G-4 was not significantly affected by gastric digestion (pH 2) – after 120 min the number of viable cells had decreased by less than 2 log units (Table I).

TABLE I. Survival of *L. fermentum* G-4 in simulated conditions of the gastrointestinal tract; CFU – colony forming units

Solution	Time of incubation min	CFU mL^{-1}	Reduction in cell viability (log units) after 120 min
Artificial gastric	0	1.21×10^{10}	
juice	60	8.90×10^{9}	
	120	2.47×10^{9}	0.69
Bile salts solution	0	3.50×10^{8}	
	60	7.30×10^{7}	
	120	1.30×10^{7}	1.43

The strain demonstrated better survival than the strain *L. casei* Shirota, where the culture exposed to gastric digestion had significant decrease of 3–4 log units (from 7.9 to 4.1) after 45 min and did survive 60 min of treatment.[23] *L. fermentum* G-4 survived in the presence of 0.5 % bile salts and showed resistance to the intestinal conditions, which corresponds to published data related to the requirement of probiotic tolerance to concentration of bile in range from 0.15 to 0.6 %.[24] It could be concluded that the strain exhibited great probiotic potential,

due to its survival and viability during exposure to simulated stomach and duodenum conditions.

MATH test/hydrophobicity

The adhesion of the isolate to hexadecane is one of the criteria for the hydrophobicity of a bacterial surface due to the absence of electrostatic interaction (caused by the large quantity of electrolytes in 0.1 M KNO_3 originating from the buffer used for cell suspension in the test). The 39±2.1 % of *L. fermentum* G-4 adhered cells indicates their intermediate hydrophobicity and correlates with previously reported data.[15] Degree of hydrophobicity of the control strain *L. rhamnosus* ATCC 7469 was 1 %. This moderate hydrophobicity could promote adequate interaction and adhesion of *L. fermentum* G-4 to gut mucosa and contribute to the beneficial effect of a potential probiotic strain.[25]

Antimicrobial activity and quantification of lactic acid

Antimicrobial activity of *L. fermentum* G-4 against different indicator strains is presented in Table II. Both fractions, complete culture and cell free filtrate, were able to inhibit the growth of *S. aureus* ATCC 6538-P, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. enteria* subsp. *enterica* serotype Abony NTCC 6017, and *C. sporogenes* ATCC 19404, but failed to inhibit *B. cereus* ATCC 11778 and *C. albicans* ATCC 10231.

TABLE II. Antimicrobial activity (diameter of the inhibition zone, mm) of *L. fermentum* G-4 against different indicator strains; BN: cell free filtrate before neutralization; AN: cell free filtrate after neutralization; n.i.: absence of inhibition; – Not tested

Indicator strain	Overnight culture, ON	Cell free filtrate	
		BN	AN
S. aureus ATCC 6538-P	18	18	0
B. subtilis ATCC 6633	12	12	0
B. cereus ATCC 11778	n.i.	n.i.	–
E. coli ATCC 8739	12	10	0
P. aeruginosa ATCC 9027	12	12	0
S. enterica NTCC 6017	12	12	0
C. sporogenes ATCC 19404	12	12	0
C. albicans ATCC 10231	n.i.	n.i.	–

The exhibited antimicrobial activities correlate with the results obtained for antimicrobial activity of *L. fermentum* strain ME-3 DSM-14241, an antimicrobial and anti-oxidative probiotic isolate with a high activity on *E. coli, Shigella sonnei*, *Staphylococcus aureus*, *Salmonella typhimurium*, and moderate activity against *Helicobacter pylori*.[26] Inhibitory effect of G-4 is completely lost when the neutralized supernatant was used, indicating that its antimicrobial activity is related to production of organic acids, primary lactic acid (Table II). Furthermore, quantitative and qualitative analysis of the filtrate (BN) by HPLC showed

the presence of lactic acid in concentration of 9.8 g L^{-1}. The detected concentration of this metabolite was higher than that of *L. acidophilus* CRL 1259 studied by Juarez-Tomas *et al*.[27]

In vivo safety evaluation of L. fermentum G-4

Abnormal toxicity test. Abnormal toxicity testing was used as confirmation for the non-toxicity of *L. fermentum* G-4. The treated group of mice was fed with lactobacilli in a dose 100 times greater (recommended single daily oral dose for mice is 1×10^7 CFU mL^{-1}) than the average of most frequently administered probiotic dose for oral use. No feeding or behavioral changes were observed in the treated mice in comparison to the control group. None of the treated mice died during the 72 h following lactobacilli administration. Therefore, the isolate most probably did not induce toxic effects, *i.e.*, it could be considered safe for oral administration.[20]

Gastrointestinal subacute local tolerance. Test of the gastrointestinal tolerance of *L. fermentum* G-4 showed that during the experiment there were no animal deaths, changes in nutrition or unusual behavior. After 7 days, the animals were sacrificed and compared with the control group. No pathological changes of the type of hemorrhage, ulceration and hyperemia were observed by macroscopic examination of the stomach and intestines. Therefore, it can be concluded that the tested bacteria were locally well tolerated.

Bacterial translocation. After the incubation period, neither *L. fermentum* G-4 nor any other microbial species could be evidenced in cultivated samples of mice blood. Obtained results confirmed that the strain was not translocated from the GIT, which makes it good candidate for further preclinical trials.

It is known that probiotics are safe for use in otherwise healthy persons, but should be used with caution in some persons because of the risk of sepsis. Newly developed probiotic strains should be thoroughly evaluated for safety before being marketed. In Finland, increased application of the probiotic *L. rhamnosus* LGG was observed since its introduction onto the market in 1990 (in 1992, over 3×10^6 kg of products containing LGG were sold).[7] This enhancement was not followed by significant increase in *Lactobacillus* bacteremia or bacteremia attributable to probiotic strains. The strain LGG and *B. animalis* subsp. *lactis* BB-12 are to date the best scientifically documented probiotics strains with more than 92 clinical trials,[28] which supports the safety of probiotics, particularly *Lactobacillus* strains.[7]

The isolated and identified *L. fermentum* G-4 expressed typical LAB phenotypic properties, absence of hemolysis, antimicrobial activity against the pathogenic bacteria indicator strains and HPLC analysis confirmed that antibacterial metabolite is lactic acid. Data obtained from *in vitro* stability tests suggest that the indigenous strains *L. fermentum* G-4 exhibit high resistance to GIT conditions, including low pH and bile salts. Furthermore, considering safety assessment, as imp-

ortant selection criterion for therapeutic use of any potential probiotic, the administered *L. fermentum* G-4 did not cause any changes in behavior and physiological activities of treated mice, no diarrheal death or its presence in their blood samples. These observations indicate that the isolate does not induce any gross acute oral toxicity on general health, growth and development of mice or poses the ability to infiltrate in areas outside the intestine.

CONCLUSIONS

The newly isolated human intestinal strain G-4 was identified as *L. fermentum*. The overnight bacterial culture showed antimicrobial potential against the selected Gram-positive, Gram-negative and sporogenic pathogenic bacterial strains due to the relatively high production of lactic acid, which enabled a rapid decrease of pH in the cultivation medium. The low degree of the reduction of cells number during simulated GIT transit classified the strain G-4 as highly resistant taking into account the previously discussed comparison with *L. casei* Shirota. According to results obtained with the MATH test, G-4 belongs to the medium hydrophobic strains. The obtained results (intestinal tolerance and antimicrobial activity, absence of hemolytic activity, toxicity and translocation) indicate that the strain G-4 could be a promising probiotic candidate, although practical application involves further *in vivo* and *in vitro* tests followed by clinical studies.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of the journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ПРОБИОТИЧКИ ПОТЕНЦИЈАЛ СОЈА *Lactobacillus fermentum* G-4 ПОРЕКЛОМ ИЗ МЕКОНИЈУМА НОВОРОЂЕНЧЕТА

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Ова студија се бави идентификовањем пробиотичког потенцијала хуманог изолата G-4, који је пореклом из меконијума. Изолат је идентификован помоћу морфолошких, физи-

олошких и биохемијских тестова и молекуларних метода заснованих на секвенцирању *16S rRNA* гена. Како би се проценила његова пробиотичка својства изведени су *in vitro* тестови: преживљавање у симулираним гастроинтестиналним условима, адхезија на хексадекан и антимикробна активност. Здравствена безбедност изолата је испитана тестирањем токсичности, гастроинтестиналне подношљивости и бактеријске транслокације *in vivo*, као и хемолитичке активности *in vitro*. Изолат G-4, идентификован као *Lactobacillus fermentum*, показао је способност преживљавања у симулираним условима желудачног сока и црева (низак степен смањења преживљавања ћелија за 0,69 и 1,3 log јединице), умерену адхезију на хексадекан (39±2,1 %), антимикробну активност према бактеријама *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serotype Abony и *Clostridium sporogenes*, услед производње млечне киселине (9,80 g L^{-1}). Изолат G-4 није испољио знаке токсичности, бактеријску транслокацију и хемолитичку активност.

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